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## **Molecular analysis of the HPJ-JT syndrome and sporadic parathyroid carcinogenesis**

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## *Chapter 9*

# Multiplex ligation-dependent probe amplification analysis in parathyroid tumours

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*manuscript*



## Abstract

### *Objective*

The objective of the present study was to develop a genomic assay based on Multiplex Ligation-dependent Probe Amplification (MLPA) for the rapid characterisation of parathyroid carcinomas based on a combination of known chromosomal amplification and deletions.

### *Patients and design*

Formalin-fixed, paraffin-embedded (FFPE) parathyroid tissues from 33 carcinoma cases and 16 adenoma cases identified in the period 1985-2003 in the Netherlands were studied. Histologically normal parathyroid tissues from 22 patients were taken from paraffin blocks and used together with a pool of 6 different normal colon appendices to serve as a reference. A MLPA probe kit was designed based on reported chromosomal amplification and deletions in parathyroid tumours.

### *Results*

Chromosomal loss in carcinomas was found on chromosome 1p (27%), 3q (21%) and 13q (21%) but was even more prominently and significantly deleted in *HRPT2* mutated carcinomas as compared to adenomas and carcinomas without a *HRPT2* mutation. Chromosome 1p, 3q and 13q showed loss in 3/5, 3/5 (both 60%) and 5/5 (100%) of the *HRPT2* mutated carcinomas, respectively.

### *Conclusion*

These results suggest that loss of chromosome arms 1p, 3q and especially 13q play a role in *HRPT2* driven tumorigenesis. Furthermore, MLPA is a useful tool to study parathyroid tumorigenesis because of the specificity/sensitivity and speed of the analysis.

## Introduction

Hyperparathyroidism is a common endocrinopathy believed to affect three in 1,000 adults<sup>1</sup> and may result from a single parathyroid adenoma (80-85%) or from hyperplasia (15-20%) but rarely (less than 1%) from carcinomas.<sup>24</sup>

Although parathyroid carcinomas are mostly slow growing, they have a high propensity (50% or more) to recur locally when not recognized at the initial surgery and treated by a simple parathyroidectomy.<sup>37</sup> Importantly, the recurrent disease is difficult to eradicate and almost 90% of all patients with recurrent hyperparathyroidism will die of the disease.<sup>20</sup> In contrast, in patients where an adequate diagnosis was made intraoperatively and who were subsequently treated by en bloc resection, local recurrence ranges from 10-33%, and long term survival improves significantly.<sup>20,38</sup>

Intraoperatively, parathyroid carcinoma usually appears as a large, firm, whitish-grey tumor that has often invaded surrounding structures. Despite these defining characteristics, parathyroid carcinoma is often not recognized at the time of initial surgery. The distinction between parathyroid carcinomas and adenomas based on histology is also difficult since the histopathological features of parathyroid carcinoma and adenoma may overlap. Some authors have claimed that trabecular growth, dense fibrous bands, spindle shape of tumour cells, mitotic figures and nuclear atypia<sup>30</sup> are helpful criteria in diagnosing parathyroid carcinomas, but all these criteria can also be observed in benign parathyroid lesions.<sup>5,23,33</sup> An unequivocal diagnosis of parathyroid carcinoma is only possible by demonstration of distant or locoregional metastasis, as well as histologically by blood vessel invasion and/or capsular invasion.<sup>29</sup> This stresses the importance of adequate

diagnosis. Therefore, there is an ongoing search for markers to provide reproducible and both biologically and clinically meaningful predictions for the diagnosis of malignancy and/or aggressive tumour behaviour that is not based on subjective histological criteria to a large degree.

So far, a variety of methods for finding and detecting molecular markers have been used, like detection of loss of heterozygosity (LOH) by microsatellite repeat analysis, comparative genomic hybridisation (CGH), immunohistochemistry (IHC) and microarray expression analysis. Results from CGH, supported by LOH studies, suggested that in those carcinomas having a physical loss of regions on chromosomes 1p (41%) and 13q (26%), there is inactivation of possible tumour suppressor genes. Chromosomal gain and thus the existence of potential oncogenes in these tumours were found in regions 1q (21%), 9q (12%) and 19p (13%).

Both CGH and LOH analysis showed that loss of 11q is a frequent event in adenomas and also in combination with *MEN1* mutations (95% in familial *MEN1* syndrome and 20-30% in sporadic adenomas). However, in a recently published paper, a high percentage (50%) of carcinomas with LOH of 11q was also detected, suggesting that it also plays a role in parathyroid carcinoma formation.<sup>16</sup>

Recently it was shown that *HRPT2* mutations are found in HPT-JT syndrome and in a substantial portion of sporadic parathyroid carcinomas, suggesting that this gene plays a pivotal role in malignant transformation of parathyroid tumours. Parafibromin encoded by *HRPT2* shows downregulation in such tumours. Furthermore, expression microarray analysis revealed that *HRPT2* mutated tumours have a unique and distinct expression profile as compared to other parathyroid tumour types. *LMNA*, *FGFR1*, *FGFR4*, *DDEF1*, *IGSF4*, *ITMB2*, *APP*, and *CDH1* are the genes that are significantly up or down regulated in the microarray analysis of a group of parathyroid carcinomas and tumours with *HRPT2* mutations. Other genes that are involved in parathyroid tumorigenesis are *CASR* and *CyclinD1* (*CCND1*).

Overexpression of the cyclin D1 protein has been demonstrated in up to 40% of parathyroid adenomas, and overexpression of PRAD1/cyclin D1, following a rearrangement with the PTH gene, has been shown in a few cases.<sup>3,18</sup> Two recent publications showed evidence that parafibromin downregulation causes an increase in CCND1 protein levels<sup>39,40</sup>. Furthermore *CASR* germline mutations can cause familial hypocalciuric hypercalcemia or neonatal severe hyperparathyroidism when partially or markedly deficient<sup>28</sup>. Mutations are also found in families suffering from FIHP.<sup>8</sup> Also, *CASR* is considered to have a potentially important secondary role in the manifestations of sporadic parathyroid tumours<sup>4</sup>, although up till now no mutations have been described in sporadic parathyroid tumours.

Multiplex ligation dependent probe amplification (MLPA) is a recently developed technique for the relative quantification of DNA sequences that can detect chromosomal deletions or amplifications.<sup>31</sup> The principle of MLPA relies on the hybridisation of sequence-specific oligonucleotides to genomic DNA, followed by ligation of the oligonucleotides and subsequent amplification of the probe. The relative peak heights or band intensities from each target indicate their initial concentration<sup>32</sup> and can be semi-quantitatively analysed.<sup>25</sup> MLPA has several advantages over currently used techniques. The first advantage is the amount of loci that can be analysed in one reaction. Furthermore, no (paired) normal tissue is needed. Finally, it is a sensitive and relatively fast technique; only a small amount of DNA is required (20 ng is sufficient for one reaction in which 40 loci are tested) and results are available within 2 days. The method is useful for archival, formalin-fixed, paraffin-embedded (FFPE) tissue as the probe target sequences are small (50-70bp).

The objective of the present study was to develop an MLPA based assay for the diagnosis of parathyroid carcinomas based on a combination of known chromosomal amplification and deletions.

## Materials and methods

### *Samples*

Formalin-fixed, paraffin-embedded tumour tissue from 28 primary parathyroid carcinomas, 4 regional lymph node metastases, and one lung metastasis taken from 30 patients was obtained from different laboratories in the Netherlands using PALGA (Dutch National Information System for Pathology, Utrecht, The Netherlands) and the archives of the Leiden University Medial Center. The samples were collected over the past 18 years (1985-2003). All but three of these samples were previously described<sup>6</sup>

Included were 30 samples with clear carcinoma features, i.e. presence of vasoinvasion and/or metastasis<sup>11</sup>, based on evaluation of representative haematoxylin and eosin stained slides of each tumour by a pathologist (HM)) and the initial pathology report. Three cases (9,11,25) were diagnosed as carcinomas based on their clinical presentation; definitive vasoinvasion was not found in these cases.

Furthermore formalin-fixed, paraffin-embedded tumour tissues from 16 parathyroid adenoma samples taken from 16 patients were obtained from the archives of the LUMC. One adenoma (48) and 1 parathyroid carcinoma (30) came from a documented HPT-JT family.<sup>17</sup>

Normal parathyroid tissues from 22 patients were taken from paraffin blocks and used together with a pool of 6 different normal appendices to serve as a reference for the Multiplex Ligation-dependent Probe Amplification (MLPA).

### *DNA extraction*

Genomic DNA from normal and tumor tissue was isolated from the paraffin-embedded material by taking tissue cores (diameter 0.6 mm) with a tissue microarrayer (Beecher) from tumor and normal areas selected on the basis of a hematoxylin and eosin-stained (HE) slide. Using a chelex extraction method, DNA was isolated from three punches, re-suspended in 96 µl of PK-1 lysis buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2.5 mM MgCl<sub>2</sub>, 0.45% NP40, 0.45% Tween 20, 0.1 mg/ml gelatin) containing 5% Chelex beads (Biorad, Hercules, California, USA) and 5 µl of proteinase K (10 mg/ml), and incubated for 12 hours at 56° C. The suspension was incubated at 100° C for 10 minutes, centrifuged at 13,000 rpm for 10 minutes, and the supernatant containing the DNA was used for PCR reactions.

### *MLPA*

MLPA has previously been described.<sup>31</sup> In brief, MLPA is based on the ligation of two DNA oligonucleotides that hybridize adjacently to a target DNA sequence. The first oligonucleotide was synthesized with, on average, a 26 bp (min: 21 bp, max: 39 bp) target-specific part and a universal M13-forward tail. The second oligonucleotide was an M13-derived single-stranded DNA sequence containing, on average, a 42 bp (min: 31 bp, max: 50 bp) target specific-part, a stuffer sequence of variable length (130-480 base pairs) and an M13-reversed tail. Thus, a probe consists of 2 oligonucleotides of which the target-specific parts hybridize adjacently and ligate. The M13 forward and reversed tails are attached to all probes, and the different length of each probe made it possible to perform a single primer multiplex PCR.<sup>25</sup>

An MLPA kit was assembled by MRC-Holland (Amsterdam, The Netherlands). Details of MLPA can be found at <http://www.mlpa.com>. The MLPA kit was designed especially/ specifically to investigate parathyroid tumours and consisted of 42 probes of chromosomal regions (based on CGH analysis<sup>2,14,21,26</sup>) and genes (based on microarray<sup>15</sup> and mutation data<sup>7,9</sup>) frequently altered in parathyroid tumours. For three important genes, we took two (*MEN1*) or three (*CASR* and *HRPT2*) different probes.

Thirty-eight experiments were performed in triplicate or more, and ten were performed in duplicate.

After denaturing 15 to 250 ng of DNA for 5 minutes at 95°C, the probe mix containing all

the probe sets was added. After overnight hybridization at 60°C, the hybridized probes were ligated for 15 minutes at 54°C with a DNA ligase. An aliquot was taken out of the ligation mix and the ligated products were amplified in a multiplex PCR reaction using forward and reverse M13 primers for 20 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C for 33 cycles in an Applied Biosystems® 9700 PCR machine. After PCR, 3 µl of the PCR products were mixed with one µL of 500 TAMRA (Applied Biosystems®) internal size marker and 20 µl deionised formamide and injected for 5 seconds in an ABI310® capillary filled with POP5 polymer. After a 30 minute run, the data were collected and analyzed with Genescan analysis and Genotyper software (Applied Biosystems®) (Figure 1). A Genotyper output file was generated combining probe set number, size and peak heights. This table was exported to a comparative access in-house adapted database where probe annotation is added to the data table. Subsequently, normalization and diagnosis of the profiles were performed.

#### *Data analysis for MLPA. Normalization.*

The MLPA traces were analyzed using the MLPAanalyzer application (<http://sourceforge.net/projects/mlpaanalyzer/>). Peak heights were dependent on sample quality, DNA concentration, hybridization parameters and instrument settings. Peaks from different probe sets also differed in magnitude in a systematic way. To normalize the raw data, MLPAanalyzer performs the following steps:

1. Distinguish focus probes and reference probes (5 loci usually unaltered in parathyroid tumours).
2. Select the reference probes from the control (non-tumour) samples. Performs steps 3 to 5 with this subset of data.
3. Within each sample divide all peak heights by the median peak height of the sample. This is to correct for the sample-to-sample variation.
4. Within each probe, divide all peak heights by the median peak height of the probe. This is to correct for systematic differences between probes. The results of 3 and 4 we call normalized peak heights.
5. Determine which of the (reference) probes are most stable. Subtract 1 from each normalized peak height and take the absolute value. Compute the median of these numbers for each probe. This is the median of the absolute deviations: MAD.
6. Select the 5 reference probes with the lowest MAD. These 5 reference probes are named calibration probes and are used to normalize the complete experiment as described in step 7 and 8.
7. Within each sample (parathyroid tumour and normal control samples), divide all peak heights by the median peak height of the 5 calibration probes of the sample of concern. This is to correct for the sample-to-sample variation.
8. Within each probe (focus and reference probes), take the median peak height of the control samples. Then, within each probe (focus and reference probes), divide all peak heights (parathyroid tumour and normal control samples) by the median peak height of the probe of concern. This is to correct for systematic differences between probes.

#### *Data visualization and interpretation.*

Each experiment was normalized and analysed separately. Scatter plots for each individual tumour and normal tissue were generated in Matlab (Figure 1) and anonymized.

To determine amplification and deletion in the analysis of the individual probes, a cut off value (amplification>1.3, deletion<0.7) was used. The evaluation of the regions was based on multiple (at least 2) probes and therefore we could use a less strict cut off; for amplification>1.2 and deletion<0.8.

To analyze the regions/chromosomal arms, we used 25 probes (region 1p: 4 probes; 1q:6 probes; 3q: 4 probes; 9p: 3 probes, 9q:2 probes, 11q: 6 probes; 13q: 5 probes). A region was considered "deleted" or "amplified" if more than 50% of the probes within

that particular region were “deleted” or “amplified” (i.e.:2/2 (100%) of the probes, 2/3 (67%) of the probes, 3/4 of the probes (75%), 3/5 of the probes (60%), 4/6 of the probes (67%)) such that they had normalized peak heights of at least 0.2 below (deletion) or above (amplification) the median normalized peak height of the reference probes.

#### *Sequence analysis*

*HRPT2* mutations and *MEN1* mutations were analysed in 27 and 23 sporadic parathyroid carcinoma samples/patients, respectively, as previously described.<sup>6</sup>

#### *LOH analysis*

From 20 parathyroid carcinoma samples, LOH status of chromosomes 1q and 11q was previously determined using microsatellite markers<sup>16</sup>

#### *IHC parafibromin*

From 27 patients, expression of parafibromin was previously determined with IHC as described<sup>35</sup>.

## **Results**

A MLPA probe set (Table 1) was constructed based on the following three criteria: a) the inclusion of genomic regions previously implicated in parathyroid tumorigenesis in the literature, such as chromosomes 1p, 1q, 3q, 9p, 9q, 11q, 13q and 19p (<sup>2,14,21,26</sup>); b) the inclusion of two crucial genes for parathyroid tumorigenesis; *HRPT2* on chromosome 1q and *MEN1* on chromosome 11q; and c) probes were included from several genes from a parathyroid carcinoma */HRPT2* genecluster as identified by cDNA expression array analysis.<sup>15</sup>

We studied 49 parathyroid tumours, 16 adenomas and 33 carcinomas. In five parathyroid carcinomas and one adenoma, somatic and/or germline *HRPT2* mutations were identified. The average amount of deletions in adenomas was 3.3 (range 0-14), the average for amplification in adenomas was 5.9 (range 0-13). Parathyroid carcinomas showed an average amount of 6.7 deletions (range 0-12) and average amount of 5.8 amplifications (range 0-19). *HRPT2* mutated samples had an average of 8.6 deletions (range 6-13) and 3 amplifications (range 1-8).

#### *Deletion and amplification of chromosomal regions*

In parathyroid carcinomas, deletion of chromosomes 1p (41%) and 13q (26%) are relatively frequently described <sup>2,14,21,26</sup>, although for chromosome 13q the frequency is only slightly increased in comparison to adenomas (Table 1). We also observed losses of these chromosomes in parathyroid carcinomas using MLPA (1p, 27.3% 9/33; 13q, 21.2% 7/33 respectively), with the losses being most notable in the *HRPT2* mutated subset of carcinomas (3/5 of 1p; 5/5 of 13q). Chromosome 13q loss was also seen for one *HRPT2* mutated adenoma. On chromosome 13q, the probes for *BRCA2* (13q12), *ITM2B* (13q14), *RB* (13q14, less clear), *DACH* (13q21) and *ING1* (13q34) were deleted in *HRPT2* mutated samples (Figure 1). Also, chromosomes 3q and 9p were deleted in a relatively high percentage of *HRPT2* mutated carcinomas (3/5 and 2/5, respectively). The most frequently found chromosomal aberration in adenomas using CGH analysis is deletion of 11q. Using MLPA, the latter was not confirmed.

Using CGH, chromosomal gains were previously found in parathyroid carcinomas of the regions 1q (21%), 9q (12%) and 19p (13%). We found in both carcinomas and in *HRPT2* mutated samples amplification of chromosome 1q in 9.1% and 1/5, respectively. Adenomas showed no amplification. MLPA of chromosome 9q did not confirm the pattern observed by CGH (amplification in 12% of carcinomas and deletion in 8% of adenomas).



TABLE 1

regions	ref CGH	ref CGH	carcinomas(n=33)*		carc with HRPT2mut (n=5)		adenomas (n=16)		ad with HRPT2 mut (n=1)	
	adenoma	carcinoma	amplification	deletion	amplification	deletion	amplification	deletion	amplification	deletion
1p	-16%	-41%	0.0	27.3	0.0	60.0	0.0	6.3	0.0	0.0
1q	-3%	21%	9.1	0.0	20.0	0.0	0.0	6.3	0.0	0.0
3q	-4%	-5%	3.0	21.2	0.0	60.0	0.0	12.5	0.0	100.0
9p	-12%	-14%	6.1	12.1	0.0	40.0	12.5	12.5	0.0	0.0
9q	-8%	12%	3.0	6.1	0.0	20.0	0.0	6.3	0.0	100.0
11q	-32%	-9%	12.1	6.1	0.0	0.0	6.3	0.0	0.0	0.0
13q	-19%	-26%	0.0	21.2	0.0	100.0	0.0	12.5	0.0	100.0
19p	-5%	13%	45.5	0.0	20.0	0.0	43.8	0.0	0.0	0.0
probes	MA	in carc								
HRPT2	no data		6.1	12.1	20.0	0.0	12.5	6.3	0.0	0.0
CASR	Down	40%	6.1	33.3	0.0	60.0	12.5	18.8	0.0	100.0
MEN1	Down	0%	9.1	9.1	0.0	0.0	18.8	0.0	0.0	0.0
LMNA	Up	100%	9.1	0.0	20.0	0.0	6.3	6.3	0.0	0.0
FGFR1	Up	90%	12.1	0.0	0.0	0.0	6.3	12.5	0.0	0.0
FGFR4	Up	70%	36.4	0.0	0.0	0.0	37.5	6.3	0.0	0.0
DDEF1	Up	80%	21.2	3.0	20.0	20.0	25.0	0.0	100.0	0.0
CCND1	Up	80%	15.2	3.0	0.0	0.0	6.3	6.3	0.0	0.0
IGSF4	Down	nd	21.2	18.2	0.0	0.0	12.5	37.5	0.0	100.0
ITMB2	Down	90%	9.1	9.1	20.0	40.0	12.5	12.5	0.0	100.0
APP	Up	90%	15.2	3.0	0.0	20.0	18.8	12.5	0.0	100.0
CDH1	up	80%	30.3	18.2	60.0	0.0	18.8	25.0	0.0	0.0

The chromosomal locations of the probes are shown on the x-axis. The y-axis shows in log scale amplification (scoring in triplicate more than 1.3), retention (around 1) and deletion (scoring in triplicate less than 0.7). Abbreviations: ref CGH: average loss of regions found by comparative genomic hybridisation analysis as reported in previous papers; MA: microarray. \* including three cases with somatic MEN1 mutations<sup>6</sup> and five cases with HRPT2 mutations. All data are percentages; the negative percentages indicate loss in the ref CGH columns, the positive percentages represent gain

Using MLPA for chromosome 19p, the observation seen in CGH (amplification in 13% of carcinomas, deletion in 5% of adenomas) was not seen with a high amplification rate in both carcinomas and adenomas.

In conclusion, using MLPA in tumours with *HRPT2* mutations, there is a significant deletion of chromosomes 1p, 3q and 13q as compared to adenomas and carcinomas without a *HRPT2* mutation ( $P < 0.05$ ).

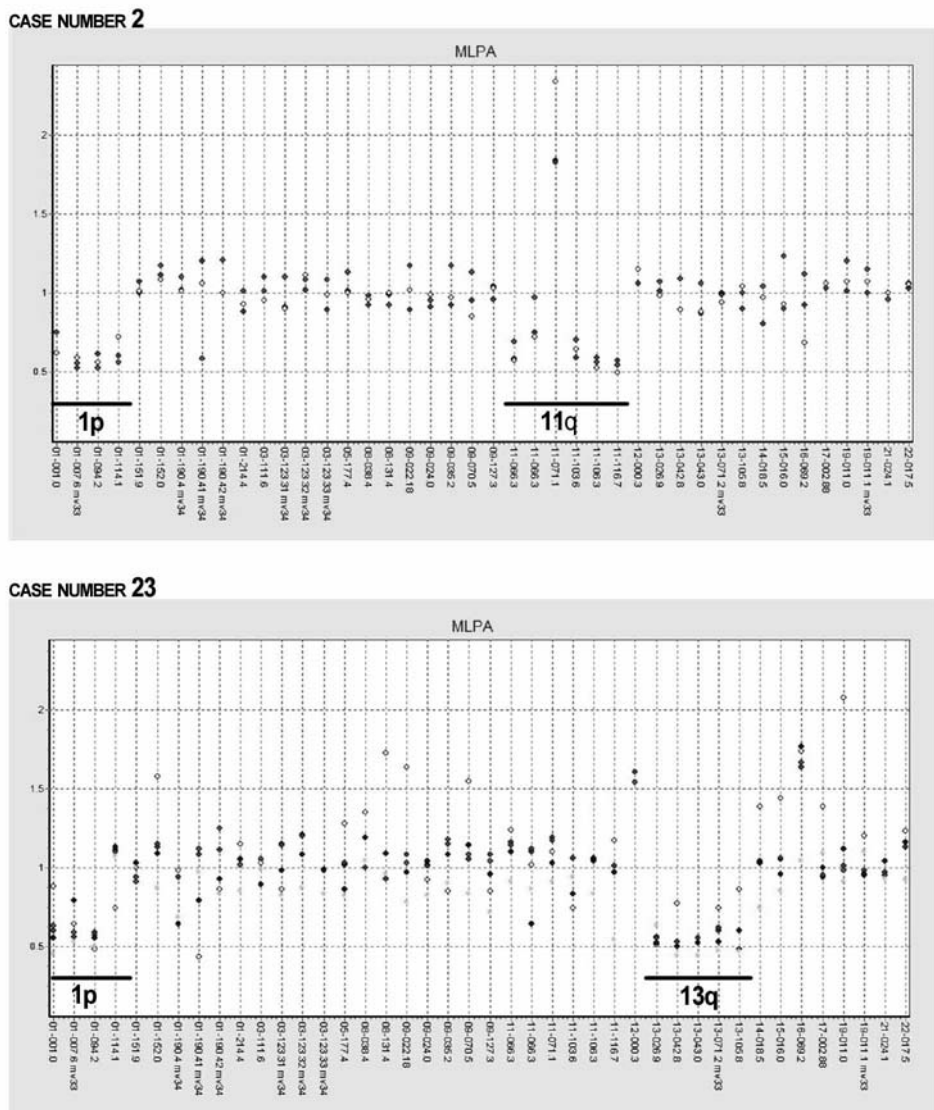
#### *HRPT2 and MEN1 MLPA*

Deletion of *HRPT2* was considered if more than 2 of the 3 *HRPT2* probes were deleted. This was the case in 3.6% (1/28) of overall carcinomas and in none of the *HRPT2* mutated samples and adenomas. Deletion of *MEN1* (in both probes) was not found in any of the adenomas and in only 9.1% of carcinomas, whereas frequently a low amplification was scored in both adenomas and carcinomas.

#### *MLPA of differentially expressed genes*

MLPA gene probes for 9 genes that were significantly up- (*LMNA*, *FGFR1*, *FGFR4*, *DDEF1*, *CCND1*, *APP* and *CDH1*) or downregulated (*CASR*, *IGSF4*, *ITMB2*) in *HRPT2* mutated samples using cDNA expression array analysis were analysed. Nonsignificant trends in the amplification/deletion of different probes were seen that mimicked the observed relative expression patterns. However, the *CASR* on chromosome 3q was scored as deleted in 33.3 % of carcinomas versus 18.8% of adenomas (nonsignificant) with frequent low amplification scores in both adenomas and carcinomas. Moreover, 4/6 *HRPT2* mutated tumours clearly showed loss of *CASR*. The trend towards amplification of *CDH1* on chromosome 16q in carcinomas and particularly in *HRPT2* mutated carcinomas (3/5) correlated with a relatively high expression of this gene.

FIGURE 1 Scatter plot of 2 parathyroid carcinoma samples.



Case no.2 (carcinoma without *HRPT2/MEN1* mutation) showed loss of region 1p and 11q. Case no. 23 (carcinoma with *HRPT2* mutation) showed loss of region 1p and 13q.

## Discussion

MLPA was used to analyse copy number variation of chromosomal regions implicated in parathyroid tumorigenesis. In the *HRPT2* mutated carcinomas, chromosomes 3q and 9p and particularly chromosome 13q showed deletions. Loss of chromosome 13q is an event found in both sporadic adenomas (19%) and carcinomas (26%), although the prevalence is higher in carcinomas.<sup>2,14,21,26</sup>

MLPA analysis showed the same trend, with the deletion of 13q found in both adenomas and carcinomas but to a greater extent in carcinomas. Remarkable is that all carcinomas and the one adenoma with a *HRPT2* mutation showed deletion of 13q. The implication that 13q deletion plays a role in malignant parathyroid tumorigenesis was previously reported by Hunt et al.<sup>19</sup> The region of loss on chromosome 13q in parathyroid tumours has been shown to include two known tumour suppressor genes, the retinoblastoma gene (*RB*, *RB1*<sup>10,12,27</sup>) and *BRCA2*.<sup>27</sup> Cryns et al<sup>10</sup> were the first to suggest that inactivation of the *RB1* gene might help to distinguish benign from malignant parathyroid tumours and thus have potential prognostic and therapeutic implications. Other authors have cast doubts on the usefulness of *RB1* gene studies in the differential diagnosis between parathyroid carcinomas and adenomas, as abnormalities of the *RB* gene and/or protein are not a specific features of parathyroid malignancy.<sup>22,27,34</sup> The above illustrates that loss of 13q is more frequently found in carcinomas than in adenomas. Although this loss is not specific for parathyroid carcinomas, it now seems to be specific for *HRPT2* mutated tumours. Additional experiments are required to further support the role of 13q in *HRPT2* driven tumorigenesis.

Chromosome 1p is the only region of significant loss common to all tumour classes. This region is the area of most frequent loss in both malignant (41%) and benign tumours (secondary (72%) and tertiary HPT (73%), and adenomas (16%)).<sup>26</sup> Hunt et al<sup>19</sup> reported that although almost all adenomas and carcinomas showed loss of markers for 1p, the benign parathyroid diseases (adenomas and hyperplasia) had a low mean fractional allelic loss (11% and 15%, respectively). The parathyroid carcinomas, in contrast, showed high mean fractional allelic loss (63%). The current MLPA analysis confirms this; we also found loss in both tumour types, but the percentage of loss and the amount of probes lost was higher in parathyroid carcinomas.

Välimäki et al (2002)<sup>36</sup> reported, furthermore, that deletion mapping studies by LOH and CGH implicate that the 1p target regions in adenomas are more distally located at 1p34-pter and are thus different from parathyroid carcinomas where the deletions cluster at 1p21-p22.

The 1p21-22 region, found to be specifically deleted in carcinomas in the study of Välimäki et al, was also more frequently lost in carcinomas than in adenomas, but the loss of the distal part of chromosome 1p in parathyroid adenomas could not be confirmed. Downregulation of *CASR* mRNA has been described in adenomas<sup>13</sup> but has also recently been shown to be downregulated and to a higher extent in the *HRPT2*/parathyroid gene cluster.<sup>15</sup> On a protein level, a strong downregulation has also been reported in parathyroid carcinomas. Up till now, no mutations have been found in sporadic adenomas, although to our knowledge no mutation analysis has been carried out on malignant tumours.

In the current MLPA analysis, deletion of 3q and especially of the *CASR* region is a frequently seen event in parathyroid carcinomas. Once again this deletion is more specific for tumours with a *HRPT2* mutation than those without, suggesting that the *CASR* might also play a role in *HRPT2* driven tumorigenesis. We now also show that downregulation of *CASR* mRNA is partly based on the physical deletion of a region of chromosome 3q containing *CASR*. Mutation analysis should further prove the complete inactivation of this gene.

Recently we showed the use of 6K SNP-arrays on FFPE material in order to detect copy number variation and copy neutral LOH. The latter information cannot be obtained using the MLPA panel. For diagnostic purposes, however, MLPA is more easily applicable.

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